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(54) Title: MUTANT MYCOBACTERIA FOR USE IN THERAPY

(57) Abstract: This invention relates to recA mutant mycobacteria, particularly mutants of mycobacterial species which are members of the Mycobacterium tuberculosis complex, such as M.bovis BCG and M. tuberculosis. These mutant mycobacteria are useful as immunotherapeutic agents and vaccines for the treatment of a range of disorders, including tuberculosis.

MUTANT MYCOBACTERIA FOR USE IN THERAPY

This invention relates to Mycobacterium mutants, particularly
mutants of mycobacterial species which are members of the
Mycobacterium tuberculosis complex, such as M.bovis BCG and M.
tuberculosis, which are useful as immunotherapeutic agents,
vaccines, or carriers for use in generating new vaccines. Such
agents are useful in the treatment of a range of disorders,
including tuberculosis.

Infection with M. tuberculosis is a major cause of human
morbidity and mortality. Despite many efforts in mycobacterial
genetics little is known about its virulence factors and
mechanisms of pathogenicity.

Mycobacterium bovis BCG is a member of the M. tuberculosis
complex which is used as live vaccine against M. tuberculosis
infection and has been administered to more than a billion
people world-wide (Cohn, D.L. (1997) Am. J. Med. Sci. 6: 372 -
376, Cohn, M.L. et al (1954) Am. Rev. Tuberc. 70: 641 - 664).
BCG has also been used as a non-specific immunotherapeutic
agent in cancer treatment (Nseyo, U.O., and Lamm, D.L. (1997)
Semin. Surg. Oncol. 13: 342 - 349; Patard, J.J. et al (1998).
Urol. Res. 26: 155 - 159). Non-virulent strains of M.
tuberculosis have also developed for use as vaccines.

BCG has a chequered history in efficacy trials. Protection
ranges between 0 and 70% and BCG shows a great deal of
geographic variability in its ability to protect against lung
tuberculosis (Fine, P. E. M. (1988) British Medical Bulletin
44: 704-716). However, in most published trials, BCG has
revealed significant protection against early childhood
tuberculosis and disseminated manifestations of the disease
(Cohn, 1997 supra).

There are several possible reasons for this variability in efficacy, including genetic differences in the host, different exposure of host to environmental non-tuberculous mycobacteria and genetic variability in BCG daughter strains.

A variety of genetic differences have been shown between daughter strains of BCG, including point mutations (Behr et al; J. Bacteriol. 2000 182:3394), variable RFLP (Behr and Small Vaccine 1999 17:915), direct repeat patterns (Howard et al J. Clin. Microbiol. 1997 35:965), variable intergenic repeats (Frothingham et al Microbiol. 1998 144:1189) and gene deletions (Behr et al (1999) Science **284**: 1520-153). The mechanisms and genes responsible for genetic instability in BCG have not been characterised. Genetic instability may be a cause of variability in the efficacy of BCG.

One of the many proteins known to be involved in DNA repair in prokaryotic and eukaryotic cells is *recA*, which regulates the error-prone DNA repair mechanism (SOS response) and is a key element of homologous recombination (Walker, G.C. (1995) *Trends Biol. Sci.* **20**: 416 - 420). The RecA of the *Mycobacterium tuberculosis* complex is known to have an unusual structure, in that it contains a protein splicing element, termed intein (Davis, E.O. et al (1991) *J. Bacteriol.* **173**: 5653 - 5662). Difficulties in achieving homologous recombination in *M. tuberculosis* complex have been attributed to this unusual structure, which may inhibit the activity of the protein, (McFadden, J. (1996) *Mol. Microbiol.* **21**: 205 - 211) although other data suggests that the *M. tuberculosis* RecA intein does not interfere with RecA function (Frischkorn, K. et al (1998) *Mol. Microbiol.* **29**: 1203 - 1214; Papavinasasundaram, K.G. et al (1998) *Mol. Microbiol.* **30**: 525-534).

Members of the *M. tuberculosis* complex, such as *M. bovis* BCG

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and *M. tuberculosis* are invasive micro-organisms which infect mammalian hosts. Phagocytic cells in a mammalian host are able to generate various chemical species including superoxide, hydrogen peroxide, and other reactive oxygen metabolites which damage microbial DNA, proteins, and membranes and present a hostile environment to invasive microorganisms (Hassett, D.J., and Cohen, M.S. (1989) *FASEB J.* 3: 2574 - 2582). Microorganisms possess various mechanisms to cope with the oxidative stress induced by phagocytes, including the *recA* dependent DNA repair system which repairs any damage resulting from this oxidative stress (Storz, G. et al (1990) *Trends Genet.* 6: 363 - 368). Such mechanisms are particularly important for the survival of intracellular pathogens in the body

DNA repair mechanisms in general and RecA function in particular have been shown to be essential for survival of intracellular pathogens by repairing DNA damage resulting from oxidative stress arising from the cellular environment e.g. in *Salmonella typhimurium* (Buchmeier, N.A., et al (1995) *J. Clin. Invest.* 95: 1047 - 1053). This is supported by evidence from *E. coli* showing that resistance to H_2O_2 correlates with the recA genotype, rather than with levels of catalase, peroxidase or superoxide dismutase (Carlsson J. and Carpenter V. (1980) 142: 319-321) and from *Erwinia carotovora*, in which the RecA-dependent SOS induction has been shown to enhance virulence (McEvoy et al (1990) *J. Bacteriol.* 172: 3284-3289).

RecA has also been generally considered to play a role in the virulence of mycobacteria, particularly intracellular pathogens such as *M. tuberculosis* (Davis, E.O. et al (1991) *J. Bacteriol.* 173: 5653 - 5662), *M. bovis* BCG and other members of the M. tuberculosis complex.

Despite its widespread use, BCG is known to cause severe infections in immunocompromised individuals (Steg, A. et al (1989) *Eur. Urol.* **16**: 161 - 164; Stone, M.M. et al (1995) *N. Engl. J. Med.* **333**: 561 - 563; Hill, A.V. (1998) *Annu. Rev. Immunol.* **16**: 593 - 617; Vesterhus, P. et al (1998) *Clin. Infect. Dis.* **27**: 822 - 825). This indicates that this organism is endowed with residual virulence properties which may manifest in the absence of an effective immune response.

The ability of BCG to survive for prolonged periods without causing progressive infection in immunocompetent individuals is an important component of its protective properties (Bloom, B.R., and Fine, P.E.M. : Bloom, B.R. (ed.) Tuberculosis: pathogenesis, protection and control. ASM Press, New York, 1994 p: 531 - 558, Behr et al (1999) supra) and in animal models, the persistence of BCG correlates with protective efficacy. It is therefore important that any *M. bovis* BCG strain used in therapeutic applications is able to survive in an immunized immunocompetent host without causing disease.

Non-virulent strains of *M. tuberculosis* may also survive for prolonged periods without causing progressive infection in immunocompetent individuals and may be used in therapeutic applications. These strains are commonly generated by the inactivation of genes responsible for virulence. The protective efficacy of these strains is also correlated with persistence in an immunized individual.

M. bovis BCG has also been proposed as an ideal delivery system for expression of foreign antigens because of this long persistence in the immunized host (Matsuo, K. et al (1990) *Infect. Immun.* **58**: 4049 - 4054; Winter, N. et al (1991) *Gene* **109**: 47 - 54; Stover, C.K. et al (1991) *Nature* **351**: 456 - 460; Aldovini, A., and Young, R.A. (1991) *Nature* **351**: 479 - 482;

Haeseleer, F. et al (1993) *Mol. Biochem. Parasit.* **57**: 117 -
126; Hess, J., and Kaufmann, S.H. (1999) *FEMS Immunol. Med.
Microbiol* **23**: 165 - 173).

M. bovis BCG and *M. tuberculosis* are slow-growing, have low
transformation efficiencies and are subject to non-homologous
genetic rearrangements. The problems posed by the genetic
manipulation of these organisms have hindered the development
of improved recombinant strains.

The present inventors have developed a novel procedure for the
generation of knock-out mutations in members of the *M.
tuberculosis* complex such *M. bovis* BCG and *M. tuberculosis*,
which is described herein. This has led to the unexpected
discovery that, in contrast to other intracellular pathogens,
the inactivation of RecA does not affect the persistence or
virulence of mycobacteria of the *M. tuberculosis* complex within
a host. Furthermore, such inactivation leads to improvements in
the genetic stability of these mycobacteria.

The term '*M. tuberculosis* complex cell' as used herein refers
to a cell of a mycobacterial species or strain which is a
member of the *M. tuberculosis* complex. Mycobacteria which are
members of the *M. tuberculosis* complex include *M. tuberculosis,*
BCG, *M. bovis, M. africanum, M. canetti* and *M. microti.*

Mycobacteria of the *M.tuberculosis* complex have endogenous
antigens which are cross-reactive with *M. tuberculosis.*
Antibodies raised against such a cross-reactive antigen will
also bind specifically to one or more antigens from *M.
tuberculosis.* These cross-reactive antigens are able to evoke
and/or potentiate an immune response against *M. tuberculosis* in
an individual. Inoculation of an individual with a
mycobacterium of the *M. tuberculosis* complex, or one or more

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antigens therefrom, primes the immune system to react against challenge with *M. tuberculosis* and may therefore potentiate a immune response against subsequent *M. tuberculosis* infection. This may reduce or abolish the symptoms and/or duration of infection and thereby provide a protective effect against such infection.

A first aspect of the present invention provides a cell of a mycobacterium which is a member of the *M. tuberculosis* complex and which has an inactivated recA function.

A cell may be in an isolated and/or purified form, i.e. separated from substances present in its natural environment, such as constituents of a culture medium.

RecA function may be reduced or, more preferably, fully inactivated in cells and compositions of the present invention.

A mycobacterial cell of the present invention persists in a host immunized therewith. The presence of the recA mutation does not affect the ability of the cell to survive in the host and the cell is able to persist in tissue without causing progressive infection in an immunocompetent host.

Preferably, a *M. tuberculosis* complex cell, particularly a M. tuberculosis cell, according to the present invention is non-virulent (i.e. the genes responsible for virulence have been inactivated) and does not evoke disease symptoms in an individual. Such a cell may be used in pharmaceutical compositions and vaccines as described herein.

An *M. tuberculosis* complex cell of the present invention may be used in a range of therapeutic, prophylactic or other medical or veterinary applications.

RecA function in a *M. tuberculosis* complex cell may be
inactivated by the inactivation of the recA gene. The recA
gene may be inactivated by a mutation such as an insertion,
deletion or frameshift mutation. Any mutation which inactivates
or reduces the activity of the recA gene may be employed in
accordance with the present invention. Mutations may occur in
the coding region and affect (i.e. reduce or abolish) the recA
activity of the expressed protein or in the non-coding region
and affect (i.e. reduce or abolish) the expression of active
recA protein.

Mutations may be induced in the recA gene of a cell by any one
of a range of conventional techniques of genetic manipulation
i.e. recombinant techniques, to produce a non-naturally·
occurring genetic sequence. Mutations may, for example, be
carried out by replacing the endogenous recA gene of an *M.
tuberculosis* complex cell, such as *Mycobacterium bovis* BCG or
Mycobacterium tuberculosis, with a recA transgene which carries
a mutation which reduces or inactivates the function of the
recA transgene.

A mutation reduces or inactivates the function of the recA
transgene may comprise the replacement of recA sequence in a
mutant recA gene with non-recA sequence. Non-recA sequence may
include a gene encoding a positive selectable marker. Other
suitable mutations are well known to those skilled in the art.
A suitable recA transgene may thus comprise exogenous or non-
natural nucleic acid sequence i.e. sequence not found in the
recA gene of a wild-type mycobacterial cell.

The endogenous gene may be replaced by a transgene using
homologous recombination, for example, using a method wherein
the cell is transformed with a vector comprising a transgene, a

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positive selectable marker and a counterselectable marker. The
transgene may comprise a gene encoding a positive selectable
marker.

Screening for recA mutants is preferably carried out using a
two stage selection. In a first stage, transformed cells in
which the vector has integrated into the genome are identified
by selecting for a positive marker on the vector. In a second
stage, transformants are then isolated in which double cross
over recombination, and therefore allelic replacement, has
occurred. The present invention encompasses host cells which
are members of the *M. tuberculosis* complex and which comprise
such a vector.

Suitable dominant negative selectable markers include rpsL and
SacB, which is preferably used in conjunction with an
additional counterselectable marker, such as a thermosensitive
origin of replication.

Other suitable methods for producing a mutant mycobacterial
cell are described in Glickman M. et al (2000) *Molecular Cell*.
5: 717-727.

A cell of a mycobacterium of the *M. tuberculosis* complex as
described herein, for example a *Mycobacterium bovis* BCG or
Mycobacterium tuberculosis cell, may be used in a method of
treatment of the human or animal body, for example a method of
therapeutic treatment.

A *M. tuberculosis* complex cell of the present invention may
further comprise a gene encoding a polypeptide which is a non-
mycobacterial or foreign antigen. Expression of such an antigen
in an *M. tuberculosis* complex cell, for example, an *M. bovis*
BCG cell allows the generation of an immune response in a

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vaccinated individual against the non-mycobacterial antigen.
The cell may therefore be used as an antigen delivery system in
the treatment of any disease, such as a pathogenic infection,
which is ameliorated by an immune response against a particular
antigen.

The improved genetic stability of mycobacterial cells of the
present invention provides for the improved retention of the
non-mycobacterial gene and therefore a more effective immune
response.

Suitable antigens include viral, protozoal, tumour cell,
bacterial and fungal antigens. For example, an antigen from H.
pylori, Measles virus (Fennelly G. J. et al (1995) *J. Infect.
Dis.* **172**: 698-705), Mumps virus, Rubeola virus (e.g. OspA:
Stover, C.K. et al (1993) *J. Exp. Med.* **178**: 197-209), B.
burgdorferi (e.g. protein A: Langermann et al (1994) *Nature*
372: 552-555), Herpesvirus, Papillomavirus, Tetanustoxin,
Diphtheriatoxin, Pneumococcus spp (e.g. Surface protein A:
Langermann et al (1994) *J. Exp. Med.* **180**: 2277-2286) tumour
cells, Leishmania (e.g. surface proteinase gp63: Connell N. et
al (1993) *Proc. Natl. Acad. Sci. USA.* **90**: 11473-11477) or HIV
(or SIV: Yasutomi Y. et al (1993) *J. Immunol.* **150**: 3101-3107)
may be used. Such an antigen may be useful in the treatment of
ulcers, measles, mumps, rubeola, Lyme disease, herpes, cancer,
tetanus, diphtheria, cancer, Leishmaniasis or AIDS
respectively.

A further aspect of the present invention therefore provides a
M. tuberculosis complex cell as described herein which
comprises genetic material encoding an antigen or immunogen
exogenous or foreign to the mycobacterium. Examples of a non-
mycobacterial antigen or immunogen that may be encoded are
listed above.

The *M. tuberculosis* complex cell is able to express the said
genetic material upon infection of a host cell, thereby
producing the encoded antigen or immunogen, to which an immune
response may be generated.

An *M. tuberculosis* complex cell of the present invention may
thereby confer immunity against a pathogen exogenous to the
mycobacterium in a susceptible species immunised therewith.

Genetic material i.e. nucleic acid encoding an antigen or
immunogen exogenous or foreign to the mycobacterium may be
introduced using techniques described herein or other suitable
techniques which are known in the art (Matsuo, K. et al (1990)
Infect. Immun. **58**: 4049 - 4054; Winter, N. et al (1991) *Gene*
109: 47 - 54; Stover, C.K. et al (1991) *Nature* **351**: 456 - 460;
Aldovini, A., and Young, R.A. (1991) *Nature* **351**: 479 - 482;
Haeseleer, F. et al (1993) *Mol. Biochem. Parasit.* **57**: 117 -
126; Hess, J., and Kaufmann, S.H. (1999) *FEMS Immunol. Med.
Microbiol* **23**: 165 - 173).

A further aspect of the present invention provides the use of a
nucleic acid comprising an inactivated recA transgene as
disclosed herein for improving the genetic stability of a M.
tuberculosis complex cell, without abolishing the virulence or
persistence of the cell. In preferred embodiments, persistence
is not affected.

The inactivated recA transgene may be used as described herein
to replace the endogenous recA gene of the cell.

A further aspect of the present invention provides a method for
improving the genetic stability of a *M. tuberculosis* complex
cell without abolishing, preferably without affecting, the

virulence or persistence of the cell, comprising inactivating a recA gene within the cell.

A related aspect of the present invention provides a method for improving the genetic stability of a vaccine comprising a *M. tuberculosis* complex cell without affecting the persistence of the cell in an individual, comprising inactivating a recA gene within the mycobacterial cell

Inactivating a recA gene may comprise replacing an endogenous recA gene with an inactive recA transgene. The replacement may occur by homologous recombination as described herein.

A *M. tuberculosis* complex cell of the present invention may be manufactured and/or used in preparation, i.e. manufacture or formulation, of a composition such as a medicament, vaccine, pharmaceutical or veterinary composition or drug. These may be administered to individuals.

Individuals include humans and other mammals, including farm animals (e.g. cows) and wild animals (e.g. badgers) which are susceptible to infection with *Mycobacterium tuberculosis*.

Pharmaceutical or veterinary compositions according to the present invention, and for use in accordance with the present invention, may include, in addition to active ingredient, a pharmaceutically acceptable excipient, carrier, buffer, stabiliser or other materials well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature of the carrier or other material will depend on the route of administration, which may be oral, or by injection, e.g. cutaneous, subcutaneous or intravenous.

Pharmaceutical or veterinary compositions for oral

administration may be in tablet, capsule, powder or
liquid form. A tablet may include a solid carrier such as
gelatin or an adjuvant. Liquid pharmaceutical compositions
generally include a liquid carrier such as water, petroleum,
animal or vegetable oils, mineral oil or synthetic oil.
Physiological saline solution, dextrose or other saccharide
solution or glycols such as ethylene glycol, propylene glycol
or polyethylene glycol may be included.

For intravenous, cutaneous or subcutaneous injection, or
injection at the site of affliction, the active ingredient will
be in the form of a parenterally acceptable aqueous solution
which is pyrogen-free and has suitable pH, isotonicity and
stability. Those of relevant skill in the art are well able to
prepare suitable solutions using, for example, isotonic
vehicles such as Sodium Chloride Injection, Ringer's Injection,
or Lactated Ringer's Injection. Preservatives, stabilisers,
buffers, antioxidants and/or other additives may be included,
as required.

Another aspect of the present invention therefore provides a
pharmaceutical or veterinary composition or vaccine comprising
an *M. tuberculosis* complex cell and having an inactivated recA
function as disclosed herein.

An inactivated recA function means an abolished or reduced recA
activity within the cell. This may be achieved by inactivating
an endogenous mycobacterial recA gene.

Such a pharmaceutical may be an immunotherapeutic agent,
vaccine, or carrier of antigenic or immunogenic material and
may be used to generate an immune response in the treatment of
a disorder in an individual in which said response is
beneficial. Suitable disorders include disorders in which an
immune response against, for example, *M. bovis* BCG or *M.*

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tuberculosis is beneficial, for example, tuberculosis and
cancer.

Another aspect of the present invention provides the use of a
M. tuberculosis complex cell as described herein in the
manufacture of a medicament for use in the treatment of a
disorder in which an immune response against an antigen
expressed by the M. tuberculosis complex cell is beneficial.

Such disorders include disorders in which an immune response
against an endogenous *M. tuberculosis* cross-reactive antigen
expressed by the mycobacterial cell is beneficial, for example,
tuberculosis and cancer, and disorders in which an immune
response against a foreign (non-tuberculosis, or non-
mycobacterial) antigen expressed by a BCG cell is beneficial
(for example, ulcers, measles, mumps, rubeola, Lyme disease,
herpes, cancer, tetanus, diphtheria, cancer and AIDS).

Another aspect of the present invention provides a method of
making a pharmaceutical or veterinary composition comprising
admixing such a substance with a pharmaceutically acceptable
excipient, vehicle or carrier, and optionally other
ingredients.

Another aspect of the present invention also provides a method
comprising the administration of a *M. tuberculosis* complex cell
as described herein to a mammal in need thereof for use in the
treatment of a disorder in which an immune response against the
cell is beneficial. Such disorders include tuberculosis and
cancer.

A *M. tuberculosis* complex cell as described herein may be used
to present foreign antigens as disclosed herein and for the
purpose of generating an immune response against the foreign

antigen. Such a cell may be used in the treatment of disorders characterised by the presence of a foreign antigen in the body, for example, infection by a pathogen.

Administration is preferably in a "prophylactically effective amount" or a "therapeutically effective amount" (as the case may be, although prophylaxis may be considered therapy), this being sufficient to show benefit to the individual. The actual amount administered, and rate and time-course of administration, will depend on the nature and severity of what is being treated. Prescription of treatment, e.g. decisions on dosage etc, is within the responsibility of general practitioners and other medical doctors.

M. bovis BCG administration is well established throughout the world as a prophylactic treatment for tuberculosis. A skilled person in the field is familiar with the protocols, formulations, dosages and clinical practice associated with the administration of *M.bovis* BCG. Such protocols, formulations, dosages and clinical practice are entirely suitable for use with pharmaceutical compositions and vaccines of the present invention.

A composition may be administered alone or in combination with other treatments, either simultaneously or sequentially dependent upon the condition to be treated.

Aspects of the present invention will now be illustrated with reference to the accompanying figures described already above and experimental exemplification, by way of example and not limitation. Further aspects and embodiments will be apparent to those of ordinary skill in the art. All documents mentioned in this specification are hereby incorporated herein by reference.

Fig. 1 shows a schematic drawing of the BCG *recA* locus: the
wildtype locus is shown along with the vector used for
inactivation, a 5′ single cross-over transformant and a knock-
out mutant. black box: cloned *recA* fragment; thin black line:
flanking genomic DNA; open box: aph-cassette; grey box: vector
backbone; hatched box: probe used for Southern blot analysis;
arrow heads and S: SmaI recognition sites.

Fig. 2 shows the survival after UV irradiation. Parental *M.*
bovis BCG (●), *recA* single cross-over transformant (■) and *recA*
knock-out mutant (▲) were irradiated with UV light for the
indicated times. Following irradiation the number of viable
cells were determined by plating.

Fig. 3 shows growth under dormancy conditions. Parental *M.*
bovis BCG (●) and the *recA* knock-out mutant (▲) were grown under
dormancy culture conditions. Growth was determined by measuring
the optical density. F and D indicate fading and complete
decolorization of the methylene blue indicator, respectively.

Fig. 4 shows survival under dormancy conditions. Survival of
parental *M. bovis* BCG and the *recA* knock-out mutant under
dormancy culture conditions was investigated by determining the
numbers of cfu after 20 days of incubation in the presence or
absence of metronidazole (10 µg/ml).

Fig. 5 shows the course of infection in Balb/c mice. Parental
M. bovis BCG (●), a *recA* single cross-over transformant (■) and
a *recA* knock-out mutant (▲) were injected into the tail vene
(approx. 10^6 cfu/animal). The number of bacteria in spleen (A)
or lung (B) were determined at different time points.

Fig. 6 shows the course of infection in nude mice. Parental *M.*
bovis BCG (●), a *recA* single cross-over transformant (■) and a

recA knock-out mutant (▲) were injected into the tail vene
(approx. 10⁶ cfu/animal). The number of bacteria in spleen (A)
or lung (B) were determined at different time points.

EXPERIMENTAL

Mutants of *M. tuberculosis* have previously been generated using
transposon mutagenesis (Camacho, L.R. et al. (1999) *Mol.
Microbiol.* **34**: 257 - 267; Cox, J.S. (1999) *Nature* **402**: 79 -
83). However, the distribution of *IS*-elements in the *M.
tuberculosis* genome is not random (Gordon, S.V. et al (1999)
Microbiology **145**: 881 - 892) and it is difficult to target
genes which are small in size.

Despite major efforts in mycobacterial genetics during the past
years, targeted gene inactivation in *M. tuberculosis* complex
remains a technical hurdle. Due to the moderate transformation
efficiencies and poor frequencies of double cross-over
recombination, the generation of knock-out mutants is laborious
and often requires the screening of numerous transformants
(Yuan, Y. et al (1998) *Proc. Natl. Acad. Sci. USA* **95**: 9578 -
9583).

The use of dominant negative selectable markers has proven a
valuable tool for targeted gene inactivation in some
mycobacteria (Sander, P. et al (1995) *Mol. Microbiol.* **16**: 991 -
1000; Pelicic, V. et al (1996) *J. Bacteriol.* **178**: 1197 - 1199;
Pavelka, M.S., and Jacobs, W.R. (1999) *J. Bacteriol.* **181**: 4780
- 4789; Pelicic, V. et al (1997) *Proc. Natl. Acad. Sci. USA* **94**:
10955-10960). *rpsL* was the first counterselectable marker
introduced into mycobacterial genetics (Sander et al., 1995
supra). This marker has been used as dominant negative
selectable marker in *Mycobacterium smegmatis*, as the *rpsL* wild-
type gene confers a streptomycin sensitive phenotype when
transformed into a streptomycin resistant strain with a mutant

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rpsL (Sander et al., 1995 supra, Sander, P et al (1996) *Mol. Microbiol.* **22**: 841 - 848; Frischkorn et al., 1998 supra).

However, the technique has not been previously applied to mycobacteria of the *M. tuberculosis* complex such as *M. bovis* or *M. tuberculosis*. Various modifications were made to the technique by the present inventors in order to generate *M. tuberculosis* or *M. bovis* BCG mutants.

Materials and Methods
DNA manipulations, isolation of plasmids
Standard techniques were used for DNA manipulation. All initial cloning procedures were performed in *E. coli* XL1-Blue MRF. Plasmids were prepared with a Quiagen plasmid preparation kit according to the manufacturers recommendations. Plasmid DNA was dissolved in TE-buffer in concentrations of 500 - 1000 ng/_l.

Cultivation of mycobacteria
When cultivated on solid medium *M. bovis* BCG strain Pasteur (ATCC 35734) was grown on Middlebrook 7H10 agar supplemented with oleic acid albumin dextrose (OADC) (Difco) for 3 - 4 weeks. Tween 80 was added to liquid broth 7H9-OADC to avoid clumping; incubation was performed in a roller bottle for 10 - 20 days. Antibiotics were added to the following concentrations: kanamycin 25 µg/ml; hygromycin 50 µg/ml; streptomycin 25 µg/ml.

Generation of suicide vectors
For the generation of suicide vectors precA::aph-rpsL and precA::hyg-rpsL the following cloning steps were performed: a 5.2 kb ApaI fragment from plasmid pEJ126 (Davis et al., 1991 supra) containing *M. tuberculosis* recA was subcloned into the PstI site of plasmid pBluescript KSII⁻ (Stratagene) resulting in plasmid pBluescript-recA. From this vector a 1.3 kbp

internal PstI fragment was substituted by a 1.3 kbp aph-
cassette isolated as a PstI fragment from plasmid pUC4K
(Pharmacia) or by a 1.8 kbp hyg-cassette isolated as a BglII-
fragment from plasmid pIJ963 (Lydiate, D.J. et al (1989) *J.*
Gen. Microbiol. **135**: 941 - 955) resulting in plasmids
precA::aph and precA::hyg, respectively. From these vectors
fragments comprising the inactivated *recA* gene were removed by
digestion with EcoRV and SpeI and cloned into ptrpA-1-rpsL
previously digested with SacI, blunt-ended and subsequently
digested with SpeI (Sander et al., 1995 supra), resulting in
suicide vectors precA::aph-rpsL and precA::hyg-rpsL,
respectively. The cloning procedures were confirmed by DNA
sequencing.

Southern Blot analyses

For Southern blot analyses 200 - 500 ng of genomic DNA were
digested with an appropriate restriction enzyme. Fragments were
separated on an agarose gel and treated according to standard
protocols with HCl, NaOH and neutralization buffer. DNA was
transferred to a Hybond-N ™ membrane (Amersham) with a vacuum
blotting apparatus and cross-linked by UV irradiation. DNA was
hybridized to *recA* probe (a 1.6 kbp ApaI/PstI fragment from
pBluescript-recA) labeled with digoxygenin according to the
manufacturers instructions (Boehringer, Mannheim), washed under
stringent conditions and developed with an antibody directed
against digoxigenin coupled with horse radish peroxidase.

Western blot analyses

M. bovis BCG strains were grown in 100 ml of Dubos broth,
induced with ofloxacin (1 µg/ml) for 24 h and cell free
extracts were prepared as described previously
(Papavinasasundaram, K.G. et al (1997) *Mol. Microbiol* **24**: 141 -
153). Cell-free extracts corresponding to 30 µg of protein, as
determined by BCA protein kit (Pierce), were separated by SDS-

polyacrylamide electrophoresis through a 10 % polyacrylamide
gel and the proteins were electroblotted onto a PVDF membrane
(Immobilon-P, Millipore). The membrane was blocked with 10 %
non-fat milk in TTBS [20 mM Tris (pH7.5), 0.5 M NaCl buffer
containing 0.1 % Tween 20] and incubated with 1:1000 dilution
of a mouse antiserum raised against purified *M. tuberculosis*
RecA protein. Mice antibody conjugated to horseradish
peroxidase (Dako) was used as secondary antibody. After washing
with TTBS, the blot was developed with diaminobenzidine reagent
solution as described previously (Davis, E.O. et al (1992) *Cell*
71: 201 - 210).

Generation of *M. bovis* BCG SMR1
M. bovis BCG (strain Pasteur) was grown in liquid medium until
an optical density of approx. 1.0. Cells were collected and
spread on 7H10-OADC agar containing streptomycin at a
concentration of 20 µg/ml. After 4 weeks of incubation at 37 °C
single colonies were picked and re-streaked on 7H10-OADC agar
containing streptomycin to confirm the streptomycin resistant
phenotype. The genotype of the streptomycin resistant strains
was determined by PCR mediated amplification of *rrs* and *rpsL*.
Sequencing of *rrs* and *rpsL* PCR products revealed a single A to
G transition in *rpsL* codon 88 conferring an amino acid exchange
from lysine to arginine.

Transformation of *M. bovis* BCG SMR1
M. bovis BCG SMR1 was grown in a 2 l roller bottle containing
400 ml 7H9-OADC-Tween until an OD of 0.6 was achieved. One day
before harvesting the cells glycine was added to a final
concentration of 1.5 % (v/v) and cells were incubated for an
additional 24 hours. All following steps were performed at room
temperature. Cells were harvested by centrifugation, washed
several times with 10 % glycerol and finally resuspended in a
volume of 5 ml. For electroporation 400 µl competent cells were

mixed with 1 µg supercoiled plasmid DNA and electroporated in a
BioRad Gene pulser II with the following settings: 2.5 kV, 1000
Ohms, 25 µF. After electroporation cells were resuspended in 4
ml of 7H9-OADC-Tween and incubated for 20 h with vigorous
shaking at 37 °C. Following incubation appropriate dilutions
were plated on selective agar. Single colonies were picked,
restreaked and grown in liquid broth when necessary.

Transformants which had undergone a homologous single cross-
over were grown in liquid broth until an optical density of
approximately 0.5 was achieved. Afterwards, appropriate
dilutions were spread on plates containing either kanamycin,
hygromycin, kanamycin plus streptomycin, or hygromycin plus
streptomycin. After 4 weeks of incubation at 37°C the
efficiency of counterselection was determined by dividing the
number of colonies obtained on plates containing kanamycin plus
streptomycin or hygromycin plus streptomycin by the number of
colonies obtained on kanamycin or hygromycin, respectively.

Construction of RecA⁻ M. tuberculosis
The suicide vector precA::hyg-rpsL was introduced into M.
tuberculosis 1424, a streptomycin resistant strain of M.
tuberculosis H37Rv containing a point mutation in the rpsL gene
(K42R), by electroporation and plated on 7H11 agar containing
50 µg ml⁻¹ hygromycin. Transformants which had undergone a
homologous single crossover were identified by Southern
analysis, grown in liquid broth to OD~0.5, and dilutions were
plated on 7H11+ hygromycin (50 µg ml⁻¹)+ streptomycin (100 µg
ml⁻¹). Colonies obtained from these counterselection plates
were tested by Southern analysis to identify those that arose
by a second crossover event. Standard blotting procedures were
used (Sambrook et al., 1989 supra) and DNA was hybridised to a
1.6kb ApaI/PstI fragment of pBluescript-recA (5' recA probe)
using the digoxygenin system according to the manufacturer's

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instructions (Boehringer, Mannheim).

Phenotypic characterization

EMS/MMS assay

7H9-OADC medium containing EMS or MMS was inoculated with 1/50
th volume of a freshly grown culture (OD 0.5). After 6 days of
incubation at 37 °C the optical density was determined. 7H9-
OADC medium without alkylating chemicals served as a control.

UV irradiation assay

For the UV-irradiation assay, 100 µl aliquots of a freshly
grown culture (OD_{600} = 0.1) were placed in an inverted lid of a
24 well culture plate and put under a standard germicidal UV
lamp (distance 20 cm). Cells were irradiated for different time
periods and samples were removed and plated on 7H10-OADC. Mean
values and standard deviations are shown from three independent
experiments. Appropriate dilutions of each culture were plated
out in duplicate.

In-vitro induced dormancy

Experiments were performed as described previously using an in
vitro dormancy model (Lim, A. et al (1999) *J. Bacteriol.* **181**:
2252 - 2256). Briefly, screw-cap test tubes (20 mm by 125 mm)
with a total fluid capacity of 25.5 ml were used. An early log
phase culture was diluted to an OD_{600} of 0.005 in a total volume
of 17 ml Dubos broth (Difco). Solid caps with latex liners were
tightly screwed down (limited oxygen supply) and the cultures
were gently stirred at 170 rpm for 20 days. Self-generated
oxygen depletion was monitored via the decolorisation of the
oxygen indicator dye, methylene blue. Growth of cultures was
monitored by OD_{600}; viable counts were determined by plating ap-
propriate dilutions on Dubos oleic album agar (Difco). When
indicated metronidazole was added at a concentration of 10
µg/ml. Mean values and standard deviations were determined from
three independent experiments. Each experiment was carried out

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with duplicate cultures. Appropriate dilutions of each culture
were plated out in triplicates.

Infections

Balb/c mice (6 - 8 weeks old) were obtained from the breeding
facility at the National Institute for Medical Research, Mill
Hill. *M. bovis* BCG strains were grown in Dubos broth.
Logarithmically growing cultures were diluted in saline to an
OD of 0.8; 0.2 ml (approximately 10^6 cfu) were injected into
the tail vein. Mice were sacrificed according to ethical
guidelines at the times indicated (three mice per BCG strain
for each time point), the spleen and lungs were removed,
weighed and homogenized. The suspensions were serially diluted
in saline and then plated on 7H10 agar supplemented with OADC.
The plates were incubated at 37°C for 3 weeks. The results were
calculated and expressed as cfu per organ.

The strain *M. bovis* BCG SMR1 (for list of strains and plasmids
see Table 1)is a streptomycin resistant derivative of *M. bovis*
BCG; this strain has a mutation in *rpsL* codon 88 Lys to Arg, a
mutation known to confer a streptomycin resistant phenotype
(Finken et al., 1993 supra).

Transformation experiments were performed with plasmid pMV361
(Stover et al., 1991 supra) and pMV361-*rpsL* (Sander et al.,
1995 supra) to investigate whether the cloned *rpsL* wild-type
gene was expressed functionally in BCG and to verify that
streptomycin resistance in BCG SMR1 is not due to additional
mutations,; pMV361-rpsL carries the *M. tuberculosis* wild-type
rpsL gene under control of its own promoter.

Transformation efficiencies were in the range of 10^4 to 2 x 10^4
for both vectors when transformants were selected on medium
containing kanamycin, the positive selection marker provided by

pMV361. In the presence of kanamycin plus streptomycin the number of colonies obtained with vector pMV361-*rpsL* was reduced 10^4-fold, whereas the number of transformants obtained with vector pMV361 was not affected.

Transformation and genetic characterization
Transformation of M. bovis BCG SMR1

M. bovis BCG SMR1 was transformed with suicide vectors precA::aph-rpsL and precA::hyg-rpsL, respectively. These vectors carry a *M. tuberculosis recA* fragment from which part of the coding region and part of the intein coding region have been substituted by either a kanamycin or a hygromycin resistance cassette. These vectors do not contain a mycobacterial origin of replication and thus are unable to replicate in *M. tuberculosis* complex. The wild-type *rpsL* gene flanking the inactivated target gene is used as counterselectable marker.

After electroporation, transformants were selected on medium containing either kanamycin or hygromycin. Transformation efficiencies when using a suicide vector in *M. tuberculosis* complex are in the range of 10 - 50 transformants per µg plasmid DNA. Eleven kanamycin resistant and four hygromycin resistant transformants were chosen at random for further investigations.

Genetic analysis of transformants and counterselection

Of the eleven kanamycin resistant clones investigated, nine clones contained the *aph* cassette indicating that these colonies arose from transformation with the suicide vector rather than representing spontaneous kanamycin resistant mutants. Genomic DNAs were isolated form these as well as the four hygromycin resistant transformants and investigated by Southern blot analyses using a *recA* fragment as probe.

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Two of the nine kanamycin resistant transformants revealed a
pattern indicating a 5´single cross-over at the *recA* locus.
After digestion with SmaI the parental wild-type strain showed
a single 2 kbp fragment. Two transformants showed a 2.6 kbp and
a 4.9 kbp fragment, respectively; these fragments represent the
vector derived, inactivated *recA* and the chromosomally encoded
functional *recA* gene. Note that the probe used did not allow to
distinguish whether the remaining transformants resulted from a
single cross-over in the 3´-flanking region or from
illegitimate recombination. Three of the four hygromycin
resistant transformants showed a pattern indicative of a
5´single cross-over. Two kanamycin and two hygromycin resistant
transformants for which a homologous single cross-over was
demonstrated were grown up in liquid broth and subjected to
counterselection on medium containing kanamycin plus
streptomycin or hygromycin plus streptomycin, respectively.
Transformants with a kanamycin plus streptomycin resistant
phenotype arose with a frequency of 10^{-4} to 10^{-5}. Hygromycin
plus streptomycin resistant transformants were observed with a
similar frequency.

Transformants were screened for the absence of the deleted
recA/intein coding sequence by PCR; seven of 40 transformants
showed a PCR result indicating *recA* inactivation. Transformants
with a *recA* deletion were grown up, and investigated by
Southern blot analysis. Following digestion with SmaI, the
kanamycin/streptomycin resistant transformants revealed a
single 2.6 kbp fragment; the absence of the 2 kbp and the 4.9
kbp gene fragments and the presence of a single 2.6 kbp
fragment indicates a second cross-over event resulting in loss
of the functional *recA* copy. The genotype was confirmed by
further genetic analyses. The hygromycin/streptomycin resistant
transformants showed a single fragment of 3.3 kbp indicative of

a *recA* inactivation.

For the further investigations one of the kanamycin inactivated *recA* mutants was chosen.

Western blot Analysis

Western blot analyses using an anti-serum raised against *M. tuberculosis* RecA was performed with the parental strain, a single cross-over transformant and the corresponding knock-out mutant. Approximately 30 µg protein was separated on a polyacrylamide gel, transferred to a PVDF membrane, and probed with an antibody raised against *M. tuberculosis* RecA. A single band of approx. 40 kDa corresponding to the mature, spliced form of *M. tuberculosis* RecA was observed in extracts of both the parental strain and the single cross-over transformant but not in the *recA⁻* mutant selected on kanamycin plus streptomycin, indicating absence of the RecA protein and thus confirming the data obtained by Southern blot analysis.

In-vitro characterization of *M. bovis* BCG *recA*

M. bovis BCG *recA⁻* strains are sensitive towards alkylating agents

One of the most noticeable phenotypes of *recA⁻* strains is their increased sensitivity towards DNA damaging agents (Kowalczykowski, S.C. et al (1994) *Microbiol. Rev.* 58: 401 - 465). To investigate the physiological effects of *recA* inactivation, several tests with different DNA damaging agents were performed. The ability of *recA⁺* and *recA⁻* strains to grow in the presence of ethylmethane sulfonic ethyl ester (EMS) or ethylmethane sulfonic methyl ester (MMS) was determined in liquid broth (Table 2).

The presence of 0.05 % EMS only slightly inhibited growth of the *recA⁺* strain. In contrast, growth of the *recA⁻* strain was

reduced 10-fold compared to that of the control. Similar
results were obtained with MMS. At a concentration of 0.006 %
MMS growth of the recA⁺ strain was not significantly affected,
whereas growth of the recA⁻ strain was inhibited by approx. 90
%. In the absence of DNA alkylating agents the growth rate of
the recA⁺ and the recA⁻ strain were indistinguishable. The
single cross-over transformant essentially behaved like the
recA⁺ strain in the presence of DNA damaging agents.

<u>M. bovis BCG recA⁻ mutants are sensitive to UV-irradiation</u>
Irradiation with UV light is frequently used to compare the
effectiveness of DNA repair mechanisms. Inactivation of the
recA gene rendered *M. bovis* BCG sensitive to UV irradiation:
the recA strain exhibited a 10.000 fold decreased viability
after 30 s of irradiation compared with a 25-fold decrease for
the recA⁺ strain (Fig. 2). The survival rate decreased
dramatically at higher irradiation dosages; only very few
survivors of the mutant strain were detected after 60 s of
irradiation (< 0.0001 %), compared to about 0.01 % survival
rate of the parental strain. These results support previous
observations that - despite the presence of an intein - *M.*
bovis BCG RecA is functionally expressed and promotes DNA
repair mechanisms in mycobacteria (Frischkorn, K. et al (1998)
Mol. Microbiol. **29**: 1203 - 1214; Papavinasasundaram et al.,
1998 supra).

<u>In-vitro induced dormancy and metronidazole resistance</u>
The obligate aerobe *M. tuberculosis* complex can enter an
anaerobic dormant state in which it survives for extended
periods of time (for review: Wayne, L.G. (1994). *Eur. J.*
Microbiol. Infect. Dis. **13**: 908 - 914). We investigated recA⁺
and recA⁻ *strains* with respect to their ability to survive in
an in-vitro dormancy model (Wayne, L.G. and Hayes, L.G. (1996)
Infect. Immun. **64**: 2062 - 2069) to determine whether the

dormancy response is affected by a loss of *recA* function.
Mycobacteria were grown in sealed and stirred tubes to achieve
self-generated oxygen depletion, a signal which triggers entry
into the dormant state. Growth of the cultures was followed by
measuring the optical density; the number of cells surviving
oxygen depletion was determined by plating. Self-generated
oxygen depletion was judged by fading/decolorization of the
indicator methylene blue.

With respect to optical density *recA*⁺ and *recA*⁻ strains behaved
identically (Fig. 3). A plateau was achieved after approx. 8
days. After day 9 and after day 15, respectively, fading and
decolorization of the oxygen indicator methylene blue was
observed in each of the cultures. After incubation for an
additional 5 days cultures were harvested and the number of
cfu's was determined (Fig. 4). The *recA* genotype did not affect
the number of viable cells after oxygen depletion indicating
that RecA does not affect the in-vitro induced dormancy
survival.

Metronidazole is the first lead compound active against dormant
M. tuberculosis. Metronidazole does not affect the growth of
aerobic grown cultures but acts exclusively on anaerobic grown
M. tuberculosis complex (Wayne, L.G., and Sramek, H.A. (1994).
Agents Chemother. **38**: 2054 - 2058).

Compared to *recA*⁺ M. bovis BCG, the *recA* mutant showed
increased susceptibility to metronidazole, i.e. the number of
viable cells was reduced about 20-fold by metronidazole for the
recA⁺ strain, but a 100-fold reduction in the number of viable
cells was observed in the *recA*⁻ mutant (Fig. 4).

Survival of the *M. bovis* BCG *recA* mutant in mice
DNA repair mechanisms in general and *recA* in particular are

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important virulence factors for the survival of intracellular
pathogens, such as *Salmonella* (Buchmeier, N.A. et al. (1993).
Mol. Microbiol. 7: 933 - 936). Although wild-type *M. bovis* BCG
does not cause a progressive infection in mice it does persist
in tissue for a significant period of time.

Balb/C mice were infected by i.v. injection with *M. bovis* BCG
recA⁺, the single cross-over transformant and the *recA*⁻ strain.
After days 1, 28, and 84, organs (spleen and lung) were
removed, homogenized, and appropriate dilutions plated on 7H10
agar. The plates were incubated for 3 weeks and the number of
cfu per organ were calculated. As shown in Fig. 5 the knock-out
mutant showed no difference in the course of infection compared
to the wild-type strain, neither in the spleen nor in the lung
of infected Balb/c mice. These data indicate that RecA is not
essential for survival in the high dose mouse infection model.
We also compared growth of the three strains in athymic mice
and again there was no significant difference in growth in
lungs or spleen.

Genetic Stability
A recombination assay selecting for drug resistance was used to
obtain demonstrate that homologous recombination is eliminated
in RecA minus *M. smegmatis*.

M. smegmatis recA⁺ and *M. smegmatis* recA⁻ were transformed with
replicating vectors (derivatives of pMV261) carrying a non-
functional fragment of rpoB using conventional procedures
(Sander et al., 1995 supra). The rpoB fragment either carried a
wild-type sequence or a mutation conferring rifampicin
resistance.

Upon recombination of the (mutated) fragment with the
chromosomally encoded rpoB gene (RNA polymerase β subunit) the

mutant fragment is incorporated into the functional gene and
confers rifampicin resistance, while the wild-type fragment
should not confer any selectable phenotype (control).

The results shown in Table 3 indicated that the generation of
the resistant phenotype was RecA dependent. Homologous
recombination in mycobacteria therefore requires the functional
RecA gene product.

Discussion

The approach described herein to transform *M. tuberculosis*
cells was similar to a strategy which has previously been used
successfully to generate allelic exchange mutants in *M.
smegmatis* (Sander et al., 1995 supra). However, as
transformation efficiencies are a critical issue, the original
procedure was modified to a two-step allelic exchange
incorporating successive steps of positive and negative
selection instead of a one-step double selection. This modified
approach overcomes reported problems with unfavourable ratios
of single cross-over transformants versus double cross-over
transformants (i.e. allelic replacement) in *M. tuberculosis*
(Yuan et al., 1998 supra).

RecA is a multifunctional and ubiquitous protein involved both
in general recombination and in DNA repair. As an inducer of
the SOS response, RecA regulates at least 20 genes, most of
which are usually suppressed by LexA (Miller, R.V., and
Kokjohn, T.A. (1990) *Annu. Rev. Microbiol.* **44**: 365 - 394).
Mycobacteria possess the key elements of a functional SOS-
system, with a LexA protein binding to a consensus sequence
GAACnnnnGTTC (Movahedzadeh, F. et al (1997) *J. Bacteriol.* **179**:
3509 - 3518; Durbach, S.I. et al (1997) *Mol. Microbiol.* **26**:
643 - 653).

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Investigations on *M. tuberculosis* RecA function have previously
been performed *in vitro*, in *E. coli* or in *M. smegmatis* (Davis
et al., 1991, 1992 supra; Davis, E.O. et al (1994) *EMBO J.* **13**:
699 - 703; Kumar, R.A. et al (1996) *Biochem.* **35**: 1793 - 1802;
Frischkorn et al., 1998 supra; Papavinasasundaram et al., 1998
supra; Vaze, M.B., and Muniyappa, K. (1999) *Biochem.* **38**: 3175 -
3186). These investigations demonstrated that the mature *M.
tuberculosis* RecA promotes DNA repair mechanisms and homologous
recombination (Frischkorn et al., 1998 supra; Papavina-
sasundaram et al., 1998 supra). However, a different,
homologous gene, e.g. *radA* (corresponding to H37Rv open reading
frame Rv 3585, Cole, S.T. et al (1998) *Nature* **393**: 537 - 544)
may compensate for RecA function *in vivo*. The present in-
vestigations provide indication that *M. bovis* BCG has a non-
redundant *recA* gene, which is essential to promote DNA repair
mechanisms.

It has recently been demonstrated that *M. bovis* BCG (Lim et
al., 1999 supra) and *M. tuberculosis* are able to enter a
dormant state. This response is triggered by slow self-
generated depletion of oxygen (Wayne, 1994 supra) Entry into
the dormant state is an adaptive process as sudden oxygen
depletion results in cell death (Wayne, L.G., and Diaz, G.A.
(1967) *J. Bacteriol.* **93**: 1374 - 1381). Although some genes
which have increased expression in the dormant phase, have been
identified e.g. α-crystallin like protein [Yuan, Y. et al
(1996) *J. Bacteriol.* **178**: 4484 - 4492] and glycin-dehydrogenase
[Wayne, L.G. and Lin, K.Y. (1982). *Infect. Immun.* **37**: 1042 -
1049]), little is known concerning the factors involved in
survival during dormancy.

Experiments in an in-vitro dormancy model are described herein.
As the number of viable mycobacterial cells after oxygen
depletion was essentially identical for the *recA*⁺ and *recA*⁻

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strain, these experiments show that RecA does not play an
essential role in entry, survival or exit from the dormant
state.

Numerous reports have demonstrated that *recA* represents an
important virulence factor: RecA is involved in stress survival
(Duwat, P. et al (1995) *Mol Microbiol* **17**: 1121 - 1131),
mediates aerotolerance in microaerophilic bacteria (Cooper,
A.J. et al (1997) *J. Bacteriol.* **179**: 6221 - 6227), induces
production of colicins, pyocins (Miller and Kokjohn, 1990
supra), and extracellular degradative enzymes (Liu, Y. et al
(1996) *Mol. Microbiol.* **22**: 909 - 918), mediates amplification
of toxin genes (Goldberg, I., and Mekalanos, J.J. (1986) *J.
Bacteriol.* **165**: 723 - 731). Most notably, *Salmonella recA⁻*
strains are highly attenuated, both in cultured macrophage
cells (Buchmeier et al., 1993 supra) and in a mouse infection
model (Buchmeier et al., 1995 supra). This effect has been
attributed to the DNA damaging effect of the oxidative burst
and the reduced ability of the mutants to perform DNA repair
(Storz et al., 1990 supra).

Our results unexpectedly show that RecA does not contribute to
the establishment and maintenance of infection in *M. bovis* BCG
or *M. tuberculosis*. This is an important finding since
persistence of BCG following vaccination is thought to be a
significant contributory factor to its immunogenicity; a mutant
BCG which is rapidly eliminated would not be an effective
vaccine.

Vaccines are the most cost-effective intervention known to
prevent disease. Viable carrier systems in general and *M.
tuberculosis* complex mycobacteria such as *M. bovis* BCG in
particular offer great potential for innovative approaches for
development of polyvalent vaccines (Matsuo et al., 1990 supra;

Winter et al., 1991 supra; Aldovini and Young, 1991 supra; Stover et al., 1991 supra). BCG is the most widely used vaccine: it can be given as a single dose at birth, and can confer long lasting immunity. *RecA* mutants may be a valuable tool for further development of *M. bovis* BCG as an antigen delivery system for expression of foreign antigens.

Table 1: Strains and vectors used

Strains

E. coli XL1-Blue	Stratagene
M. bovis BCG strain Pasteur	ATCC 35734
M. bovis BCG SMR1	this study
M. bovis BCG recA$^+$/recA::aph	this study
M. bovis BCG recA$^+$/recA::hyg	this study
M. bovis BCG recA::aph	this study
M. bovis BCG recA::hyg	this study

Plasmids

pMV361	Stover et al., 1991
pMV361-rpsL	Sander et al., 1995
pEJ126	Davis et al., 1991
pUC4K	Pharmacia
pIJ963	Lydiate et al., 1989
pBluescript SKII- (Stratagene)	E. coli cloning vector
ptrpA-1-rpsL	Sander et al., 1995
pBluescript-recA	This study
pBluescript-recA::aph	This study
pBluescript-recA::hyg	This study
precA::aph-rpsL	This study
precA::hyg-rpsL	This study

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Table 2: Growth in the presence of alkylating agents

	recA$^+$	recA$^+$/recA$^-$	recA$^-$
7H9	0.687	0.653	0.679
+ EMS (0.05 %)	0.374	0.376	0.077
+ MMS (0.006%)	577	0.608	0.098

Growth as determined by optical density (OD600 nm) of parental
recA$^+$, single cross-over transformant (recA$^+$/recA$^-$) and recA$^-$
mutant in 7H9-OADC medium containing EMS [0.05 % (v/v)] or MMS
[0.006 % (v/v)]. Cultures were inoculated with approximately
10^6 cfu/ml and incubated for 6 days.

Table 3. Frequencies of mutation to rifampicin resistance by recombination

Strain	plasmid	mean relative frequency[c]
M. smegmatis recA[+]	rpoBwt[a]	1.0
M. smegmatis recA[+]	rpoBmut[b]	145.0
M. smegmatis recA[−]	rpoBwt	0.8
M. smegmatis recA[−]	rpoBmut	0.9

[a] wt = wild type rpoB fragment

[b] mut = mutated rpoB fragment

[c] relative to the background; spontaneous frequency of
     mutation to rifampicin resistance

CLAIMS:

1.   A cell of a mycobacterium which is a member of the *M. tuberculosis* complex and which has an inactivated recA function

2.   A cell according to claim 1 which is a *Mycobacterium bovis* BCG or *Mycobacterium tuberculosis* cell.

3.   A cell according to claim 1 or claim 2 which is non-virulent.

4.   A cell according to any one of the preceding claims wherein the recA gene of said cell is inactivated by mutation.

5.   A cell according to any one of the preceding claims which comprises genetic material encoding an antigen or immunogen exogenous or foreign to the mycobacterium.

6.   A cell according to claim 5 wherein said antigen is a viral, protozoal, tumour cell, bacterial or fungal antigen.

7.   A cell according to any one of the preceding claims for use in a method of treatment of the human or animal body.

8.   Use of a cell according to any one of the preceding claims in the manufacture of a medicament for use in the treatment of a disorder in which an immune response against an antigen expressed by the M. tuberculosis complex cell is beneficial

9. Use according to claim 8 wherein said disorder is a mycobacterial infection.

10. A method for improving the genetic stability of a *M.*

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tuberculosis complex cell without affecting the persistence of
the cell in a host, comprising inactivating a recA gene within
the cell.

11.   A method according to claim 10 comprising replacing the
endogenous recA gene of said cell with a recA transgene which
carries a mutation which reduces the function of the recA
transgene.

12.   A method according to claim 10 or claim 11 comprising
formulating said cell into a pharmaceutical preparation.

13.   A pharmaceutical composition comprising an *M. tuberculosis*
complex cell according to any one of claims 1 to 7.

14.   A method of making a pharmaceutical composition comprising
admixing a cell according to any one of claims 1 to 7 with a
pharmaceutically acceptable excipient, vehicle or carrier.

15.   A method comprising the administration of a cell according
to any one of claims 1 to 7 to a mammal in need thereof for use
in the treatment of a disorder in which an immune response
against the cell is beneficial.
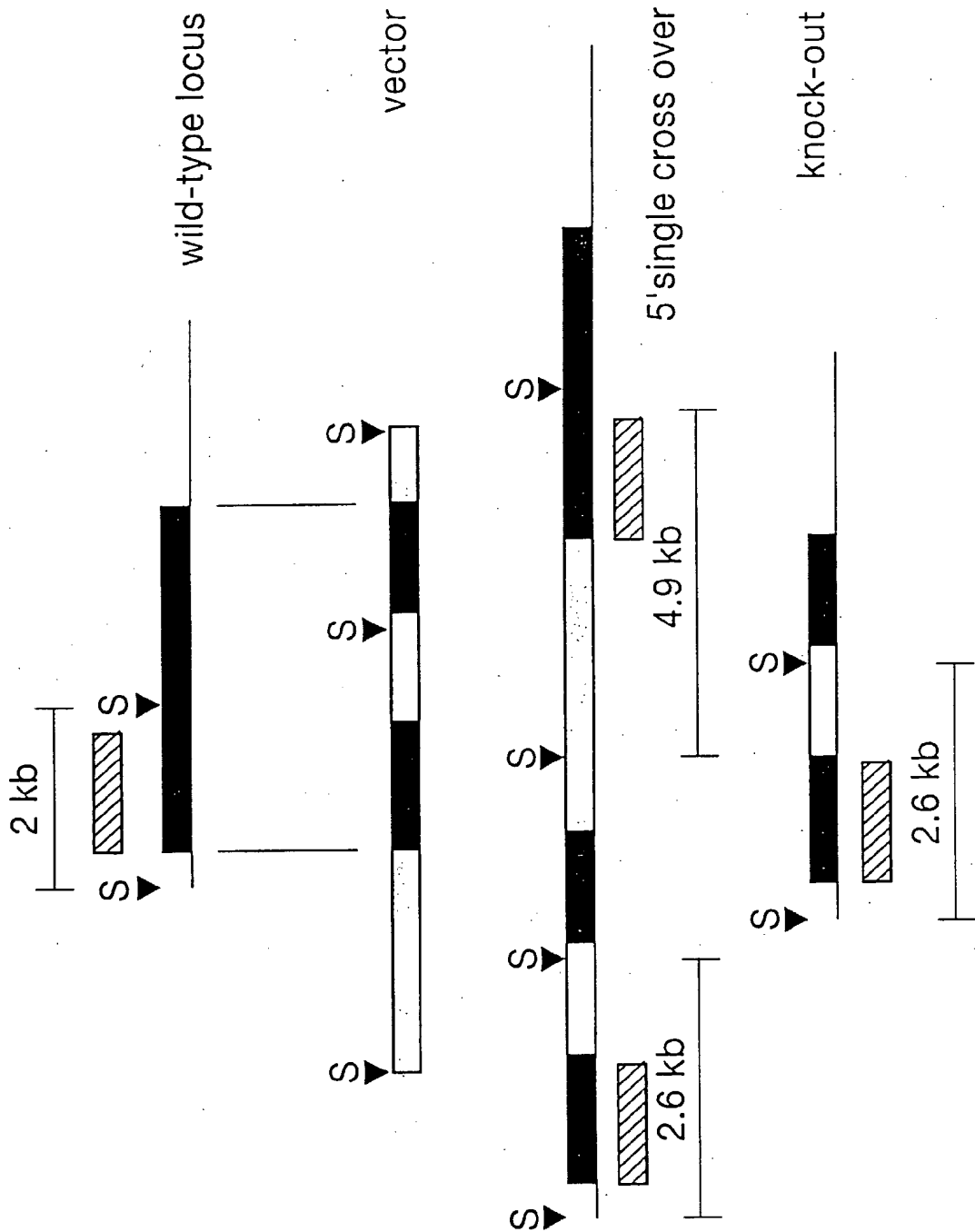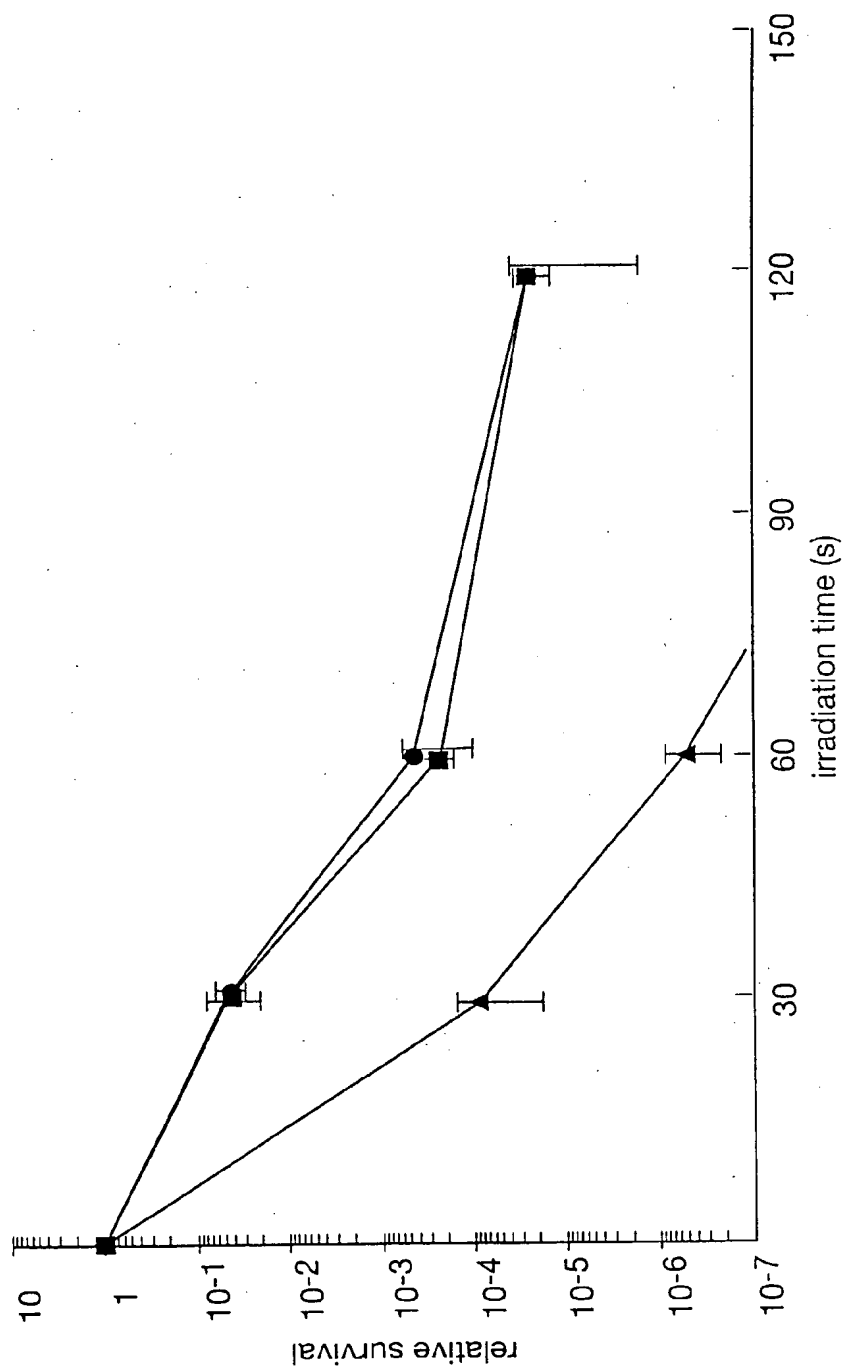
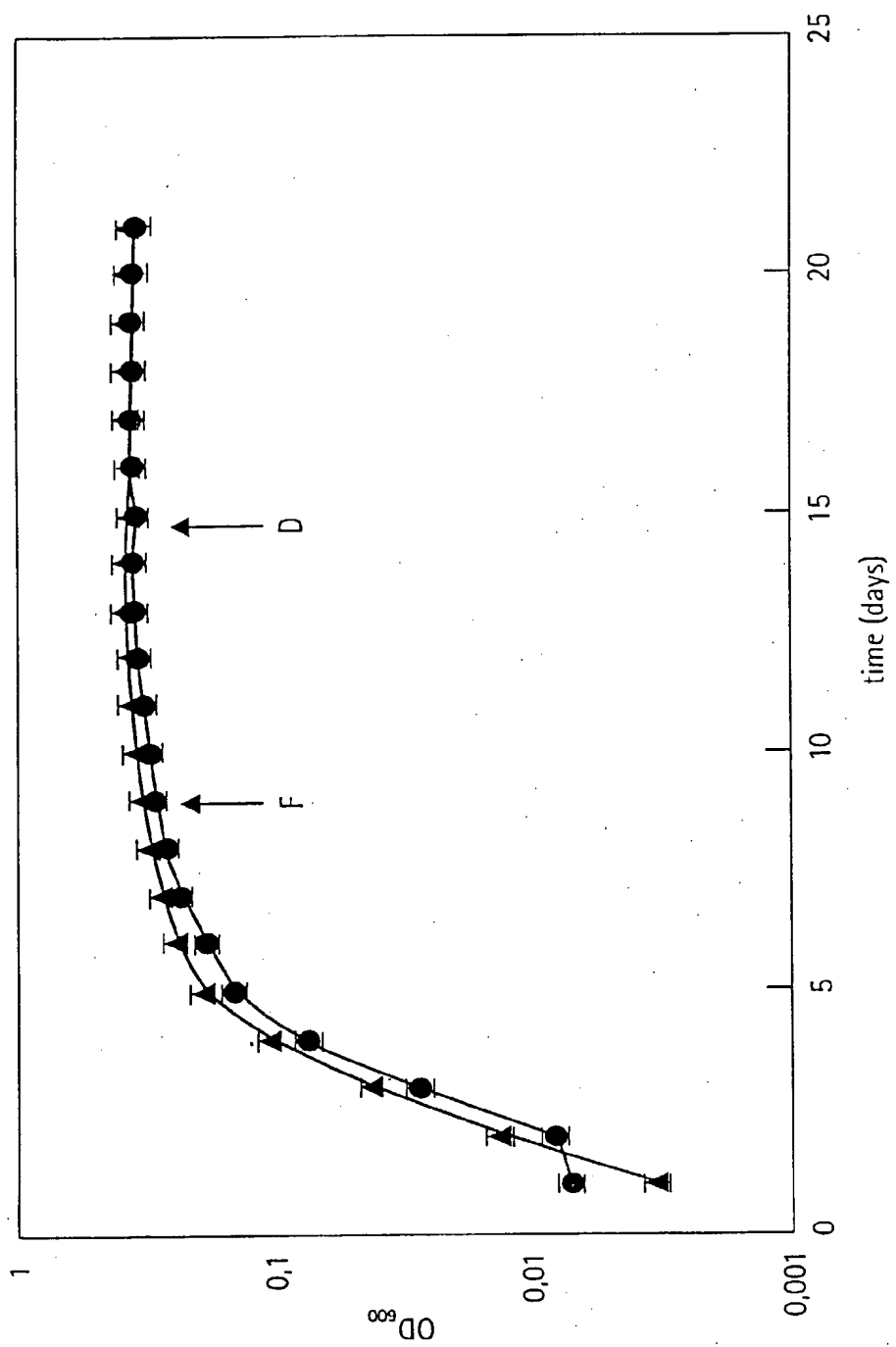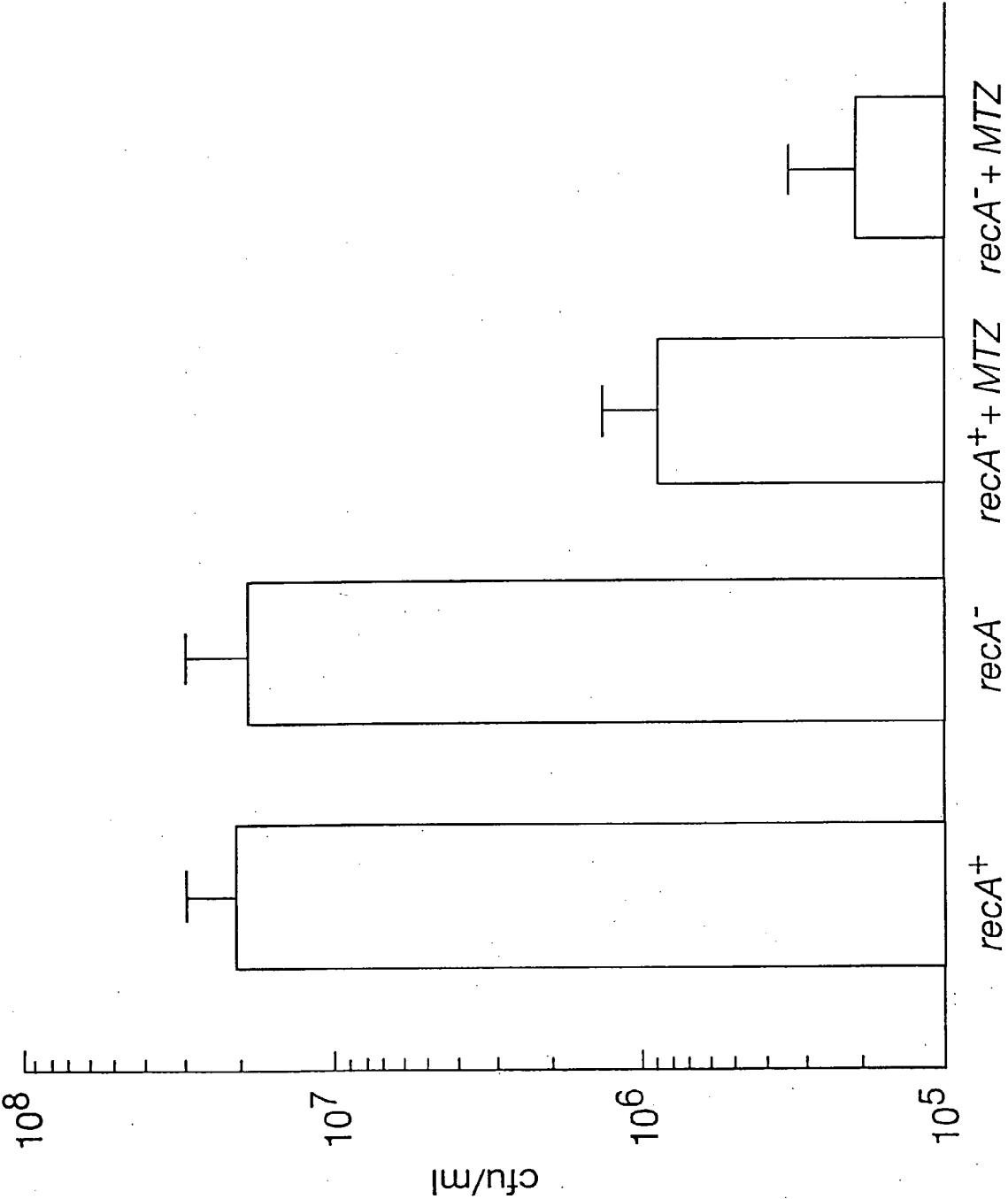16. A method according to claim 17 wherein the disorder is a
mycobacterial infection.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6